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SOME KINETIC PROPERTIES OF HUMAN RED CELL UROPORPHYRINOGEN DECARBOXYLASE

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Summary

Several kinetic properties of uroporphyrinogen decarboxylase (uroporphyrinogen-III carboxy-lyase, EC 4.1.1.37) from human hemoglobin-free hemolysates were studied, using substrates of both isomeric series I and III (uroporphyrinogen, hepta and pentacarboxyl porphyrinogens). Enzyme affinity for series III isomers was always found to be higher than for corresponding series I isomers.

Mixed substrate experiments using porphyrinogen (both labelled with ^{14}C and unlabelled) showed:

(a) a reciprocal inhibition of decarboxylation of series III porphyrinogens by series I porphyrinogens with the same number of carboxylic groups;

(b) no inhibition of hepta- and pentacarboxylic series III porphyrinogens decarboxylation by uroporphyrinogen III.

It is demonstrated that porphyrinogens of both isomeric series with the same number of carboxylic groups are decarboxylated at the same active center; in contrast, the sequential decarboxylation of uroporphyrinogen III to coproporphyrinogen III occurs at four different active centers. Relationship between the kinetic properties of uroporphyrinogen decarboxylase and biological data of porphyria cutanea are discussed.

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Abbreviations: 7-COOH-porphyrinogen, heptacarboxylic porphyrinogen; 5-COOH-porphyrinogen, pentacarboxylic porphyrinogen.

Introduction

Uroporphyrinogen decarboxylase (uroporphyrinogen-III carboxy-lyase, EC 4.1.1.37) catalyses the sequential decarboxylation of the four acetate substituents of uroporphyrinogen I and III to form coproporphyrinogen [1,2]. This enzyme was partially purified from bacterial [3], avian [4] and mammalian sources [5]. Recent studies have shown that the decarboxylase from human red cells is a single protein [6,7]; it was also demonstrated that decarboxylation of uroporphyrinogen I occurs by a nonspecific route in contrast to the highly ordered 'clockwise' decarboxylation of uroporphyrinogen III [8,24]. The decarboxylation could occur in a two stage process involving initially a rapid elimination of the first carboxyl group from uroporphyrinogen and then a slower elimination of the further three carboxyl groups [4]. It remained unclear, however, whether these reactions were catalyzed at the same active center or not. The purpose of our study was to determine some kinetic properties of human erythrocyte uroporphyrinogen decarboxylase. We show that this enzyme has four catalytic sites (one for each porphyrinogen), there is reciprocal competition between isomers I and III (with the same number of carboxyl groups) for the same site and nonreciprocal enzyme inhibition by porphyrinogens of the same isomeric series but with a different number of carboxyl groups. These results are discussed in relation to the biological data of porphyria cutanea, a disease characterized by a deficiency of uroporphyrinogen decarboxylase in liver [9,10] and, in some cases, in erythrocytes [7].

Materials and Methods

(1) Chemicals were obtained from the following sources: δ-[4-^{14}C]aminolevulinic acid (specific activity 50 Ci/mol) was purchased from Amersham (U.K.); unlabelled δ-aminolevulinic acid and Mesoporphyrin IX (dimethyl ester) were from Sigma (France); Aquasol was from New England Nuclear (F.R.G.). All other chemicals used were of reagent grade and were obtained from the usual commercial sources. 5-COOH-porphyrinogen III was a gift from Prs. G.H. Elder and A.H. Jackson (Cardiff, U.K.).

(2) The porphyrin methyl esters were analyzed by high-pressure liquid chromatography (HPLC) as previously described [7], using a Perkin-Elmer chromatograph (model 604) with a 30 × 0.4 cm 10 μm Porasil Column (Waters). The 404 nm chromatogram was recorded by using an LC 55 Perkin-Elmer detector. Peak areas were determined with a computing integrator (icap 5, LTT, France).

Erythrocytes were isolated from heparinized blood (1 ml) by Blincoe's method [11]. Red cells were then lysed by freezing and thawing twice. Proteins in the hemolysate were applied on a DEAE-cellulose suspension (Whatman DE 52) as described by Henessey et al. [12]. Hemoglobin was discarded by three washings with 5 mM sodium phosphate buffer (pH 7.0) and proteins were eluted with 0.5 M KCl. The hemoglobin-free hemolysate allowed us to study uroporphyrinogen decarboxylase without further purification, because in erythrocytes this enzyme is the last in the heme biosynthesis pathway; complicated manipulations aimed at discarding heme after incubation were avoided.

The amount of protein present (0.8—1.4 mg/ml) were determined by the procedure of Lowry et al. [13].

(3) Substrates

(a) Uroporphyrin I and other porphyrins of isomeric series I. Uroporphyrin I (octamethyl ester) was obtained from Sigma (France) or prepared from the urine of a patient with congenital erythropoietic porphyria. 7-COOH-, 6-COOH-, and 5-COOH-porphyrins were obtained by decarboxylation of uroporphyrin I following the method of Edmondson and Schwartz [14] slightly modified: uroporphyrin I was heated at 176°C during 30—45 min in sealed tubes in vacuo. The porphyrin methyl esters were separated and purified by thin-layer chromatography (TLC) as already described [15].

(b) Uroporphyrin III and other porphyrins of isomeric series III. Uroporphyrin III was enzymatically synthetized from δ-aminolevulinic acid using a technique already described [15] with slight modification: briefly, 30 ml of hemoglobin-free human hemolysate in 0.5 M KCl were added to 70 ml of Tris-HCl buffer (100 mM, pH 8.6), containing reduced glutathione (0.1 mM) and δ-aminolevulinic acid (0.15 mM), and incubated for 8 h in the dark at 37°C.

7-COOH-porphyrin III was synthetized using the same procedure, but at pH 7.9. The incubation was stopped with 200 ml of ethyl acetate/acetic acid (3 : 1, v/v) and the esterification was performed as previously described [15]. Porphyrin esters were separated by TLC on silicagel (1 mm Schleicher and Schull G_{1510} LS_{254}) in the modified system of Elder [16] containing carbon tetrachloride/dichloromethane/ethyl acetate/ethyl propionate (2 : 2 : 1 : 1, v/v). The uroporphyrin or 7-COOH-porphyrin area was scraped off into a tube and the porphyrins were eluted from the silica with 10 ml of chloroform/methanol (5 : 1, v/v); the solvent was evaporated under reduced pressure and the porphyrin was then chromatographed again in the same mixture. After these two chromatographies, contamination between uroporphyrin and 7-COOH-porphyrin was less than 1% as measured in HPLC. Porphyrin esters were hydrolysed with HCl 8 M for 48 h, dried in vacuo over KOH and dissolved into NH_4OH/0.1 M to give 0.05—0.2 mM solution. Porphyrins were conserved at 4°C in the dark.

Control of isomeric composition. About 0.5 nmol of porphyrin was diluted with 2 ml HCl 0.5 M and decarboxylated by the method of Edmondson and Schwartz [14]. Isomers of coproporphyrin were separated according to Jensen [17]. Porphyrins were located under ultraviolet light and after scraping the silica and eluting with 0.6 ml of a 1 M perchloric acid/methanol (1 : 1, v/v) mixture, fluorescence was recorded using a semi micro cell holder in a Hitachi Perkin Elmer 204 (excitation 400 nm, emission 595 nm). Percentage determination showed that uroporphyrin III and 7-COOH-porphyrin III were less than 5% contaminated with isomer I. Uroporphyrin I was found to be pure, containing less than 5% of isomer III.

(c) Preparation of [^{14}C]uroporphyrin III and 7-COOH-[^{14}C]porphyrin III. [$^{14}C_8$]Uroporphyrin and [$^{14}C_8$]heptacarboxyl porphyrin were synthesized enzymatically from δ-[4-^{14}C]aminolevulinic acid using the incubation medium described for non-labelled porphyrins: δ-[4-^{14}C]aminolevulnic acid was diluted with unlabelled aminolevulinic acid to obtain a specific activity of 2.5 Ci/mol

(so that the specific activity of porphyrins formed was 20 Ci/mol). After purification, chemical purity was tested on HPLC and radiochemical purity was tested on TLC in the modified solvent system of Elder [16] on silica gel thin-layer plastic plates (0.24 mm, Schleicher and Schüll F 1500). More than 98% of radioactivity was found on the plate at the same place as the fluorescence of the corresponding porphyrin ester. Porphyrin esters were hydrolysed and stored as described above.

(4) Measurement of porphyrinogen decarboxylase activity

(a) Preparation of porphyrinogens. Porphyrins were reduced to porphyrinogens with 3% (w/w) sodium amalgam (following the technique of Falk [18]) and diluted with an equal volume of 250 mM potassium phosphate buffer (pH 6.7) containing 30 mM sodium thioglycollate. Substrate concentration (0.05—0.2 mM) was determined spectrophotometrically in 0.5 M HCl after reoxidation.

(b) Incubation. The standard assay mixture contained in a final volume of 2 ml, 1.6 ml potassium phosphate buffer (250 mM, pH 6.7) with disodium EDTA (0.1 mM), 0.2 ml of enzyme preparation and 0.2 ml of porphyrinogen solution (concentration indicated in the legends); the final pH was 6.75 ± 0.05. In mixed substrate experiments, both porphyrins (one ^{14}C-labelled and one unlabelled) were reduced separately and an appropriate volume of each porphyrinogen solution was added to the assay mixture without exceeding a total volume of 0.2 ml. In all experiments a reagent blank (no enzyme added) was included. Each tube was kept cold in an ice-bath and was flushed with N_2 before and during the addition of porphyrinogens, then sealed under N_2 with a rubber cap. The reaction was started by bringing the tubes to 37°C in the dark usually for 30 or 60 min. The reaction was stopped by adding 8 ml of ethyl acetate/glacial acetic acid (3/1 v/v) containing 1 nmol of mesoporphyrin IX as an internal standard. After mixing, the tubes were left in the light for 30 min to allow oxidation of porphyrinogens to porphyrins. Porphyrins were converted into their methyl esters as already described [15].

(c) Separation and measurement of formed products. Porphyrin esters were dissolved in 50 µl of chloroform:

20 µl were injected into HPLC, separated and quantified spectrophotometrically as previously described [7,23]; in a few experiments, the fractions obtained were collected in glass vials (2—6 ml) and the solvent evaporated at 45°C in an airstream. Aquasol (10 ml) was added to each vial and radioactivity was then measured as described below.

20 µl of the same solution were streaked on silicagel thin-layer plastic plates and chromatographed with porphyrin ester markers (from eight to four carboxyl groups) in the solvent system of Elder [16]. After a first development, the plate was allowed to dry, and the labelled substrate area (uroporphyrin or heptacarboxyl porphyrin) was cut off into a glass vial; the plate was chromatographed once more in the same solvent system; each porphyrin ester area was then cut off into a glass vial, mixed with Aquasol (10 ml) and left 20 min for elution before counting the radioactivity.

Radioactivity was measured by using the ^{14}C channel of a LS 30 Intertechnique liquid scintillation spectrometer (France).

Separation of labelled products from labelled substrates was usually done on TLC plates and not by HPLC, because separation on TLC plates with two migrations allowed a still lower contamination (less than 1%) than HPLC.

(d) Calculation of enzyme activity. Uroporphyrinogen decarboxylation was measured as (hepta + hexa + penta + copro) porphyrinogen formed; in the same way, 7-COOH-porphyrinogen decarboxylation was measured as (hexa + penta + copro) porphyrinogen formed and 5-COOH-porphyrinogen decarboxylation as coproporphyrinogen formed. In experiments where unlabelled porphyrinogen I and [^{14}C]porphyrinogen III were used together as substrates, decarboxylation of labelled substrate was estimated from the radioactivity (corrected with counting efficiency) of products separated on TLC plates; decarboxylation of unlabelled substrate was calculated as being the difference between the spectrophotometric determination of formed products (corresponding to isomers I and III) and the corresponding radioactive determination (isomer III).

When two substrates of isomeric series III were incubated together, the substrate with the highest number of carboxylic groups was ^{14}C-labelled; the decarboxylation of the labelled substrate was estimated as described and the decarboxylation of the other substrate was estimated spectrophotometrically by HPLC.

Enzyme activity is expressed as units per mg of protein (1 unit:1 nmol of porphyrinogen(s) formed per h at 37°C) after correction for recovery which was estimated as follows: when HPLC was used to separate the products, an internal standard (mesoporphyrin IX) was used; when ^{14}C-labelled products were separated on TLC plates, recovery was determined by comparing the quantity of substrate in the incubation medium with the sum of substrate and different products recovered from TLC.

(e) Measurement of K_m and K_i. K_m was determined using the Lineweaver and Burk plot; the lowest concentrations of substrates used were around 0.3 μM. In these conditions less than 30% of the substrate was consumed at the end of the incubation; according to Lee and Wilson [25], the arithmetic average substrate concentration during the incubation time was used in place of initial value to calculate K_m.

K_i was measured as follows. In mixed substrate experiments with porphyrinogens of both isomeric series, by using the formula:

$$K_i = \frac{[I]vK_m}{[S]V - [S]v - vK_m} \qquad \text{(Table II)}$$

In mixed substrate experiments with porphyrinogens III either with the above formula (Table III) or by using the data plotted in Figs. 3—5 (intercept of the straight line on the $1/S$ axis).

Results

Fig. 1 shows the variation of the enzyme activity with time using 5-COOH-porphyrinogen III or uroporphyrinogen III as substrate. The initial concentration of substrates was about 8 K_m; after 90 min, 30—40% of both substrates was consumed and final concentration was about 5 K_m. The graph of 5-COOH-porphyrinogen III decarboxylation is linear but the graph of uroporphyrinogen

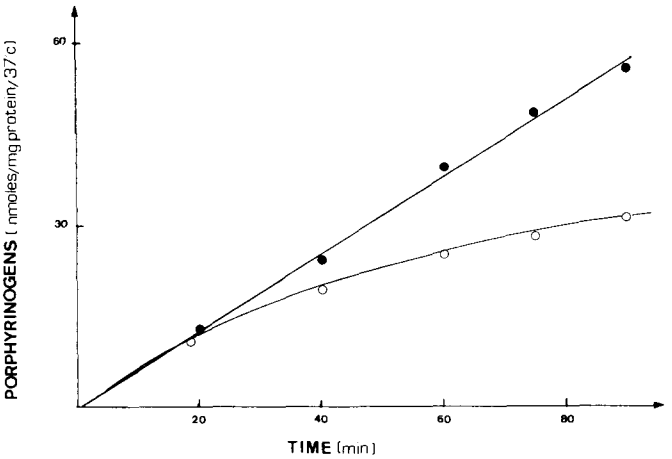

Fig. 1. Time course of porphyrinogen decarboxylase activity. •, decarboxylation of 5-COOH-porphyrinogen III. ○, decarboxylation of uroporphyrinogen III. Substrate concentration was 3.3 μM. Hemoglobin-free human hemolysate (0.3 mg of protein) was used as the enzyme source.

III curves downward. This cannot be explained by the decrease in substrate concentration and suggests an inhibition by the products formed.

The amount and the percentage of 7-COOH-porphyrin III and uroporphyrin III obtained from δ-aminolevulinic acid with human hemolysate are dependent on the pH of the medium of incubation: at pH 8.6, uroporphyrin is the most important product (more than 95% isomer III); at pH 7.9, whereas uroporphyrin is a mixture of isomer III (75%) and isomer I (25%), 7-COOH-porphyrin is only of type III; at pH 7.4, 7-COOH-porphyrin is still 75% type III, whereas uroporphyrin is now only 25% type III. Coproporphyrin is always type III. These results confirm the data of Cornford [19] and strongly suggest a selection of isomer III by the uroporphyrinogen decarboxylase.

Table I shows the activity of uroporphyrinogen decarboxylase (V) and the

TABLE I

UROPORPHYRINOGEN DECARBOXYLASE ACTIVITY (V) AND K_m FROM HEMOGLOBIN-FREE HUMAN HEMOLYSATE WITH SUBSTRATES OF ISOMERIC SERIES I AND II

The substrate concentrations used to measure enzyme activity (V) were 8—10 times higher than K_m. K_m is expressed as the mean from at least two assays. n, number of assays.

Substrate	n	Activity (V) (nmol porphyrinogen/h per mg protein)	K_m (μM)
Uroporphyrinogen III	3	25.7 (24.7—28.6)	0.4
Uroporphyrinogen I	15	13.3 ± 2.5 (mean ± S.D.)	1.0
7-COOH-porphyrinogen III	3	9.95 (8.0—11.9)	0.35
7-COOH-porphyrinogen I	3	29.1 (25.9—32.4)	0.85
5-COOH-porphyrinogen III	3	42.9 (35.1—51.8)	0.4
5-COOH porphyrinogen I	35	4.80 ± 0.6 (mean ± S.D.)	0.9

different K_m obtained with Uro-7-COOH- and 5-COOH-porphyrinogens of both isomeric series: uropophyrinogen III is decarboxylated more quickly than uroporphyrinogen I. In contrast decarboxylation of 7-COOH-porphyrinogen III is almost three times slower than decarboxylation of the corresponding isomer I. Table I shows also that enzyme affinity is always higher for isomers III (K_m = 0.35—0.40 μM) than for isomer I (K_m = 0.85—1 μM).

It is well known that using uroporphyrinogen III as substrate, the two main products obtained are 7-COOH-porphyrinogen and coproporphyrinogen [4,20], 7-COOH-porphyrinogen being accumulated in greater amounts than the other intermediates. Fig. 2 shows that with low concentrations of substrate (1 μM) the percentage of coproporphyrinogen obtained is higher than the percentage of 7-COOH-porphyrinogen, whereas the reverse is obtained with high concentrations (10—15 μM).

Studies with substrates of both isomeric series

(a) Results of experiments using both isomers I and III of uroporphyrinogen in the same incubation are shown in Table II. When decarboxylation of uroporphyrinogen III was measured, the presence of uroporphyrinogen I inhibited the reaction with a K_i around 1.5—2 μM (assuming that the inhibition is competitive and using a K_m of 0.4 μM for uroporphyrinogen III). When uroporphyrinogen I was considered as the substrate, its decarboxylation was also inhibited by uroporphyrinogen III; a K_i around 0.3—0.55 μM was found using a K_m of 1 μM for uroporphyrinogen I.

(b) In the same way, a reciprocal inhibition was found between both 7-COOH-porphyrinogen isomers (Table II); using a K_m of 0.35 μM for 7-COOH-porphyrinogen III, a K_i of approx. 1.45 μM was calculated for 7-COOH-porphyrinogen I (mean of three assays); using a K_m value of 0.85 μM

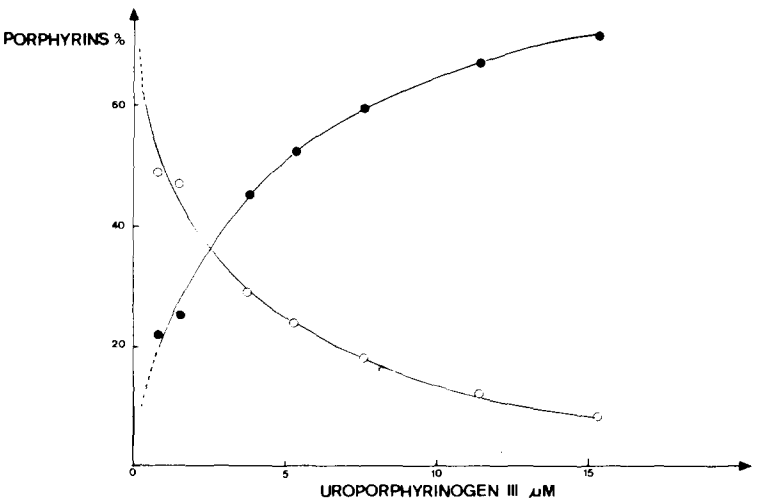

Fig. 2. Effect of uroporphyrinogen III concentration on 7-COOH-porphyrinogen and coproporphyrinogen formation. Incubations were carried out at 37°C for 1 h as described in Materials and Methods, except that the concentration of the potassium phosphate buffer was 100 mM. •, 7-COOH-porphyrinogen formed; ○, coproporphyrinogen formed.

TABLE II

UROPORPHYRINOGEN DECARBOXYLASE ACTIVITY: RECIPROCAL INHIBITION BY BOTH ISOMERS I AND III OF UROPORPHYRINOGEN AND 7-COOH-PORPHYRINOGEN

The concentration of isomer I is higher than the concentration of isomer III, but in fact the ratio isomer concentration/K_m is similar for both isomers.

Substrate	Inhibitor	Activity (nmol of porphyrinogen/h per mg protein)
Uroporphyrinogen III (μM)	Uroporphyrinogen I (μM)	
	none	25.5 */26.6 **
5.9	18.5	15.8
	37	9.2
	none	28.7 */29.8 **
11.8	18.5	22.3
Uroporphyrinogen I (μM)	Uroporphyrinogen III (μM)	
	none	13.5 *
18.5	5.9	8.9
	11.8	4.7
	none	14.5 *
37	5.9	10.7
7-COOH-porphyrinogen III (μM)	7-COOH-porphyrinogen I (μM)	
	none	7.3 */7.4 **
1.08	4.3	3.3
	8.6	2.55
2.16	none	8.0 */8.5 **
	4.3	6.2
7-COOH-porphyrinogen I (μM)	7-COOH-porphyrinogen III (μM)	
	none	25.1 *
4.3	1.08	14.4
	2.16	11
	none	26.7 **
8.6	1.08	15.5

* Activity was measured using the spectrophotometric method.
** Activity was measured using the radiochemical method.
These notes apply also to Table III.

for 7-COOH-porphyrinogen I, a K_i of approx. 0.25 μM was found for 7-COOH-porphyrinogen III (mean of four assays).

Studies with substrates of the same isomeric series

(a) [^{14}C]Uroporphyrinogen III and 7-COOH-porphyrinogen III. At high concentrations ($K_m \times 6$—30) a reciprocal inhibition between both porphyrinogens was not found: whereas uroporphyrinogen III decarboxylation was strongly inhibited by 7-COOH-porphyrinogen III (Table III), the decarboxylation of 7-COOH-porphyrinogen III was not affected by the presence of uroporphyrinogen III (Table III). Variations of uroporphyrinogen III (substrate) and 7-COOH-porphyrinogen (inhibitor) concentrations revealed that inhibition of the decarboxylation of uroporphyrinogen III appears to be competitive (Fig. 3); K_i was about 0.15 μM (mean of three assays).

TABLE III

UROPORPHYRINOGEN DECARBOXYLASE ACTIVITY: EFFECT OF 7-COOH-PORPHYRINOGEN ON UROPORPHYRINOGEN III DECARBOXYLATION AND OF UROPORPHYRINOGEN III ON 7-COOH- AND 5-COOH-PORPHYRINOGEN DECARBOXYLATION

Substrate	Inhibitor	Activity (nmol of porphyrinogen/h per mg protein)
Uroporphyrinogen III (μM)	7-COOH-porphyrinogen III (μM)	
	none	28.3 */29.6 **
5.9	3.05	15.2
	6.1	7.9
11.8	none	33.1 */31.9 **
	3.05	21.4
7-COOH-porphyrinogen III (μM)	Uroporphyrinogen III (μM)	
	none	6.55
3.05	5.9	7.3
	11.8	6.60
	none	6.5
6.1	5.9	6.2
5-COOH-porphyrinogen III (μM)	Uroporphyrinogen III (μM)	
	0.27	42.4
	0.46	40.1
0.9	0.92	40.3
	2.3	39.7
	0.27	51.2
	0.46	51.8
1.8	0.92	52.3
	2.3	52.0

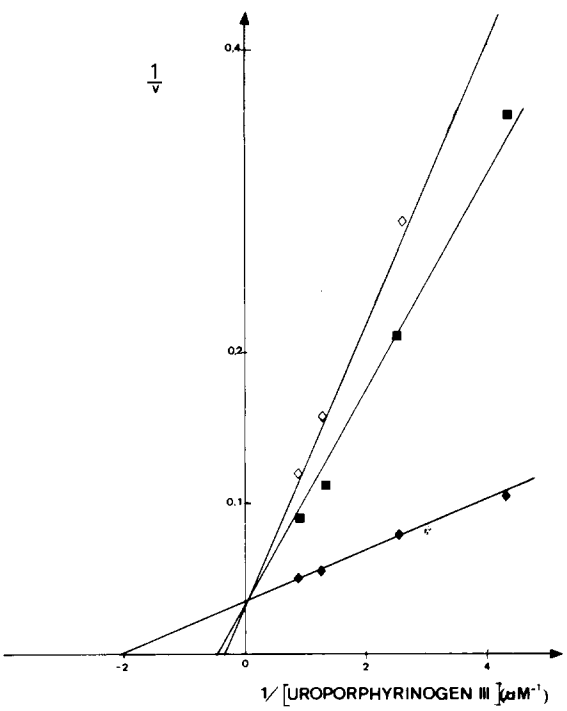

Fig. 3. Effect of 7-COOH-porphyrinogen III on [$^{14}C_8$]uroporphyrinogen III decarboxylation. Measurements were made without inhibitor (♦) and at two concentrations (0.42 μM, ■; 0.85 μM, ◇) of 7-COOH-porphyrinogen III. The total volume of (substrate + inhibitor) solution was adjusted to 0.2 ml with 0.1 M NH_4OH treated as described for porphyrin substrate solution. Other experimental details are given in

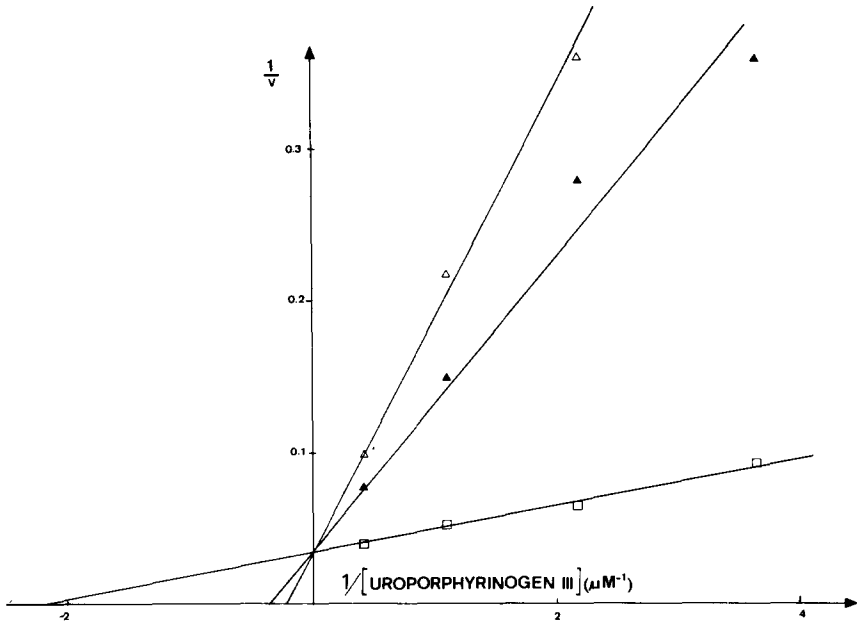

Fig. 4. Effect of 5-COOH-porphyrinogen III on [$^{14}C_8$]uroporphyrinogen III decarboxylation. Measurements were made without inhibitor (□) and at two concentrations (0.9 μM ▲; 1.8 μM △) of 5-COOH-porphyrinogen III. Other details are given under Fig. 3.

(b) [^{14}C]uroporphyrinogen III and 5-COOH-porphyrinogen III. 5-COOH-porphyrinogen III was found also to be a competitive inhibitor of the decarboxylation of uroporphyrinogen III (Fig. 4); K_i was about 0.17 μM (mean of three assays). In contrast, uroporphyrinogen III did not inhibit decarboxylation of 5-COOH-porphyrinogen III (Table III).

(c) 7-COOH-[^{14}C]porphyrinogen III and 5-COOH-porphyrinogen III.

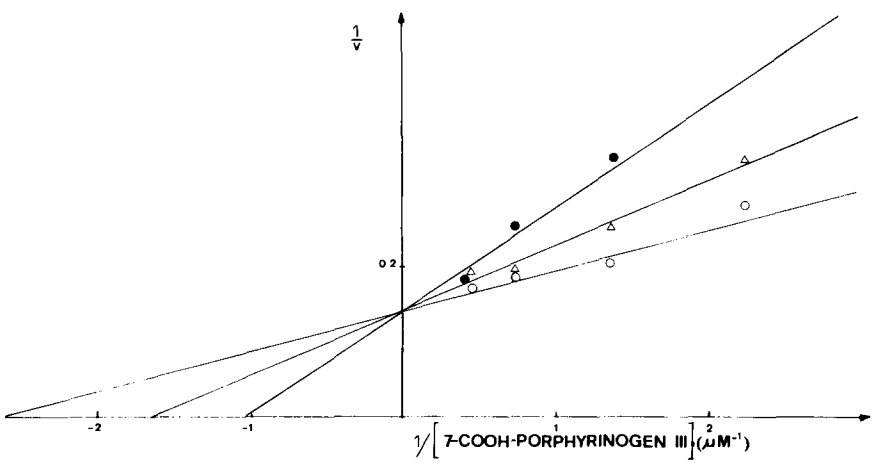

Fig. 5. Effect of 5-COOH-porphyrinogen III on 7-COOH-[$^{14}C_8$]porphyrinogen III decarboxylation. Measurements were made without inhibitor (○) and at two concentrations (0.28 μM △; 0.56 μM ●) of 5-COOH-porphyrinogen III. Other details are given under Fig. 3.

Decarboxylation of 7-COOH-porphyrinogen III is inhibited by the 5-COOH-porphyrinogen III, whereas the reverse is not found. Fig. 5 shows that the inhibition is competitive, the K_i was found to be about 0.18 μM (mean of four assays).

Discussion

Porphyrinogen decarboxylase activity from human hemoglobin-free hemolysate was studied with different substrates from isomeric series I and III. Data with series III isomers (Table I) are in agreement with previous reports [6,20]: decarboxylation of uroporphyrinogen III is nearly three times faster than decarboxylation of 7-COOH-porphyrinogen III; decarboxylation of 5-COOH-porphyrinogen III is the fastest, in keeping with the finding that 7-COOH-porphyrinogen and coproporphyrinogen are the most important products when uroporphyrinogen is used as substrate and that very small amounts of pentacarboxylic porphyrinogen are found.

Comparisons between activities with isomers I and III show that decarboxylation of uroporphyrinogen III is twice as fast as decarboxylation of uroporphyrinogen I (Table I) according to the first results of Mauzerall and Granick [1] with avian erythrocytes. In contrast to our data, identical V values have been found with both isomers [5,21], from mouse spleen and human erythrocytes, respectively. Present K_m values are of the same order of magnitude as those reported by Romeo and Levin [5]: 0.9 μM for uroporphyrinogen III and 1 μM for uroporphyrinogen I. However, in contrast with these autors, we have found a significantly lower K_m for isomers III than for isomers I. These discrepancies could be methodological in origin since these authors used enzymatically generated substrates in the incubation mixture. Anyway, it seems that there is a good correlation between our data and the biochemical findings in sporadic porphyria cutanea: the excessive amounts of porphyrins in urine and feces, particularly uroporphyrin I and 7-COOH-porphyrin III [22] are well explained by (a) the selection of isomer III at the first step of decarboxylation of uroporphyrinogen and perhaps also (b) a higher V with 7-COOH-porphyrinogen I as substrate at the second step.

Several lines of evidence show that successive decarboxylation from uroporphyrinogen (I or III) to coproporphyrinogen are catalyzed in human erythrocytes by a single enzyme.

(1) Enzyme activity towards uroporphyrinogen III and 5-COOH-porphyrinogen III was copurified from human erythrocyte haemolysate to an approximately 500-fold increase in specific activity [6].

(2) Recently, we have shown that a 50% decrease in uroporphyrinogen decarboxylase activity in hemolysates from subjects with familial porphyria cutanea could similarly be found using 8-, 7-, 6- or 5-COOH-porphyrinogen as substrate [7]. It was not clear whether these different decarboxylations occur at the same active center or not; Tomio et al. [4] suggested on the basis of kinetic data that the enzymic molecule contains at least two active centers.

Our present results (Table III) show in mixed substrate experiments that uroporphyrinogen does not inhibit decarboxylation of either 7-COOH-porphyrinogen or 5-COOH-porphyrinogen; 7-COOH-porphyrinogen does not inhibit

decarboxylation of 5-COOH-porphyrinogen, implying that 5-COOH-porphyrinogen, 7-COOH-porphyrinogen and uroporphyrinogen are decarboxylated at different active sites on the enzyme. One may postulate that there are four sites, one for each decarboxylation (direct evidence of a distinct site for decarboxylation of 6-COOH-porphyrinogen was not obtained because of lack of availability of this substrate).

In contrast, 5-COOH- and 7-COOH-porphyrinogen competitively inhibit decarboxylation of uroporphyrinogen (Fig. 3 and 4) and 5-COOH- also competitively inhibits decarboxylation of 7-COOH-porphyrinogen (Fig. 5).

These findings suggest that a less carboxylated substrate can competitively occupy the active site of substrates with a higher number of carboxylic groups (without being decarboxylated) but, in contrast, a more carboxylated substrate cannot fit into the active site of a substrate with a lower number of carboxylic groups.

It is surprising that the K_i found for porphyrinogens that inhibit decarboxylation of uroporphyrinogen seem to be smaller than their corresponding K_m as substrates; this finding could be explained by the fact that the actual concentration of inhibitor was higher than the concentration deduced from the amount of inhibitor added to the incubation mixture, because some inhibitor is generated by decarboxylation of the substrate during incubation. This hypothesis is in keeping with the nonlinearity of uroporphyrinogen decarboxylation with time (Fig. 1) (the coproporphyrinogen formed does not seem to be an inhibitor since 5-COOH-porphyrinogen decarboxylation was linear with time).

Results of mixed-substrate experiments, using either uroporphyrinogen I and III or 7-COOH-porphyrinogen I and III show a reciprocal inhibition between these substrates. The K_i calculated for each isomer considered as a competitive inhibitor is in good agreement with its own K_m value as substrate. These results support the hypothesis that isomers I and III with the same number of carboxylic groups are decarboxylated at the same active site.

Only small amounts of 5- and 6-COOH-porphyrinogens are found in vitro when uroporphyrinogen III is used as the substrate and coproporphyrinogen is the most important product when the concentration of uroporphyrinogen is low; these facts suggest that intermediates between uroporphyrinogen and coproporphyrinogen are not entirely released into the incubation medium, at least a fraction being directly transferred from one decarboxylation site to the next. One may consider the possibility that in vivo, where substrate concentrations are usually low, all intermediates remain bound to the enzyme, explaining the fact that little or no accumulation of intermediates occurs. However, in porphyria cutanea, it is surprising that the porphyrin accumulated in the liver and excreted in the urine or feces consists not only of uroporphyrin but also 7-COOH-porphyrin [22]; if the enzymatic defect involves each step of decarboxylation, one would expect only the excess of uroporphyrin. Nevertheless, this porphyrin excretion pattern could be explained by the data shown in Fig. 2, since the higher the uroporphyrinogen concentration, the greater is the percentage of 7-COOH-porphyrinogen obtained; 7-COOH-porphyrinogen in excess would be oxidized in the cell and then leaves the metabolic pathway.

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